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A Sympathetic View on Free Radicals in Diabetes

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Neuropathies are severe complications of diabetes. In this issue of *Neuron*, Campanucci et al. report that hyperglycemia-induced elevation of reactive oxygen species impairs synaptic transmission of sympathetic neurons leading to diabetes-induced dysautonomias. These observations provide new insights into the etiology of diabetic complications and suggest potential novel therapeutic approaches for neuropathies.

Neuropathies are important comorbidities of type 2 diabetes. Depending on the affected nerves, symptoms of diabetic neuropathy can include pain and numbness in the extremities and problems with the digestive system, urinary tract, blood vessels, and heart. The symptoms can be mild but may also be debilitating, even severe and sometimes fatal (Freeman, 2005). While there is consensus that diabetes-associated hyperglycemia leads to these various types of neuropathies, the molecular underpinnings of impaired neuronal function triggered by diabetes are ill-defined.

In this issue of *Neuron*, Campanucci et al. (2010) provide evidence that an elevation of cellular reactive oxygen species (ROS) during hyperglycemia plays an important role in the depression of synaptic transmission in sympathetic neurons, which may be responsible for the development of neuropathies. Synaptic transmission in sympathetic efferents occurs via activation of nicotinic acetylcholine receptors (nAchR) in sympathetic ganglions, such as the superior cervical ganglion (SCG). These ionotropic receptors are heteromultimers consisting of different components, including α, β, γ, and δ subunits. Upon acetylcholine (Ach) binding, nAChR activation enables cation (primarily sodium) entry to the cytosol, leading to membrane depolarization, which increases excitatory postsynaptic potentials (EPSP) and elevates excitatory neurotransmission (Figure 1). In this study, Campanucci et al. first showed that in the streptozotocin-induced diabetes model, in which insulin-producing pancreatic beta cells are eliminated, Ach-evoked EPSPs were significantly depressed in sympathetic neurons of the superior cervical ganglia (SCG) compared to nondiabetic control mice. This decreased excitatory neurotransmission in diabetic mice was associated with a reduction in sympathetic nerve activity leading to decrease in heart rate and loss of thermoregulation. To test whether the overall outcome of the intervention is due to diminished insulin levels (loss of pancreatic beta cells) or elevated circulating glucose levels (the consequence of insulin depletion), they tested sympathetic activity in animals with naturally occurring diabetes, mice that have no circulating adipose-derived leptin (ob/ob mice) or long form of leptin receptors (db/db mice). In these animals, hyperglycemia is associated with high levels of insulin. In both ob/ob and db/db mice, depressed synaptic transmission in the sympathetic nervous system was observed. Since alterations in synaptic transmission were observed both in type I (lack of insulin) and type II (elevated insulin) diabetic models, their findings argue for the role of hyperglycemia rather than insulin in this pathophysiological process.

To understand the mechanism by which hyperglycemia could induce a depression in synaptic transmission, the authors used an in vitro approach. First, they showed that exposure of cultured neonatal SCG neurons to high glucose levels induced an increased staining of 4-hydroxy-2-nonenal (HNE), a major product of endogenous lipid peroxidation. Then, to test whether this hyperglycemia-induced ROS elevation causes a use-dependent inactivation of nAChRs, they recorded from SCG neurons with whole-cell voltage clamp using a high (25 mM) and a lower (5 mM) concentration of glucose in the media. When neurons where exposed to high glucose levels, repeated application of Ach caused a use-dependent rundown of the Ach-evoked current compared to control neurons maintained at lower glucose levels. To test whether ROS are responsible for this effect, neurons were then treated with antioxidants such as α-lipoic acid and catalase and Ach-evoked currents were recorded. Pretreatment of neurons with antioxidants prevented the rundown of the Ach-evoked currents in neurons exposed to high glucose levels, thus suggesting that hyperglycemia-induced ROS were responsible for the detrimental effect on nAChRs function.

To understand the putative molecular mechanism of the inactivation of nAChRs by ROS, Campanucci et al. studied the known effect of ROS on the oxidation of cysteine (Cys) residues on the α subunit of nAChRs. They transfected sympathetic neurons of α3 KO mice (nonfunctional nAChRs) with either a vector expressing the α3 wild-type receptor ($\alpha3^{WT}$) or with a mutated receptor in which the Cys at position 239 in the α3 subunit was replaced by an alanine residue ($\alpha3^{C239A}$). When $\alpha3^{WT}$ neurons were exposed to high glucose levels, repeated Ach application caused the rundown of the ACh-evoked currents. However, when the $\alpha3^{C239A}$-transfected neurons were

exposed to high glucose levels, the Ach-evoked currents were stable, indicating that the inactivation of the nAchR by hyperglycemia-induced ROS production involves the Cys at position 239 of the α3 subunit. Finally, to test whether the Cys residue was responsible for the depression of synaptic transmission in vivo, they infected α3 KO mice with adenoviral vectors expressing either the $\alpha3^{WT}$ or the $\alpha3^{C239A}$, induced diabetes by STZ injection, and compared the nerve-evoked EPSPs in SCG neurons. In diabetic α3 KO mice infected with $\alpha3^{WT}$, the nerve-evoked EPSPs were significantly reduced 2 weeks after the onset of diabetes. However, in diabetic α3 KO mice infected with $\alpha3^{C239A}$, the nerve-evoked EPSPs were not different than those of nondiabetic WT mice. In addition, while the diabetic α3 KO mice infected with $\alpha3^{WT}$ showed a reduced sympathetic drive of the heart and had impaired thermoregulation in cold, the diabetic α3 KO mice infected with $\alpha3^{C239A}$ showed no differences in these sympathetic adaptations.

The adaptive process of sympathetic activation enables an organism to utilize its internal energy sources to launch a response in self defense to support survival. Beyond the aforementioned effects on heart rate and thermoregulation, both of which enable efficient and rapid transport of nutrients to the muscles and brain, increased sympathetic tone promotes acute gluconeogenesis and glucose release from the liver to provide an internal source of fuel. While these adaptive responses are very reasonable at the time of predatory threat or fasting, when reliance on internal energy sources for survival is critical, to maintain such a sympathetic tone during diabetes would contribute to elevated circulating glucose levels. In most cases of type 2 diabetes, hyperglycemia is the consequence of both overnutrition and increased hepatic glucose production and release. Thus, from this perspective, a sustained hyperglycemia-induced decrease in the sympathetic outflow, the focus of the paper, may actually be seen as a positive adaptation whereby it is an attempt to decrease liver production and release of glucose to diminish hyperglycemia. It remains to be seen whether restoration of the sympathetic outflow in diabetes, as being proposed by Campanucci et al. (2010), would actually be beneficial or detrimental for glycemic control in diabetes.

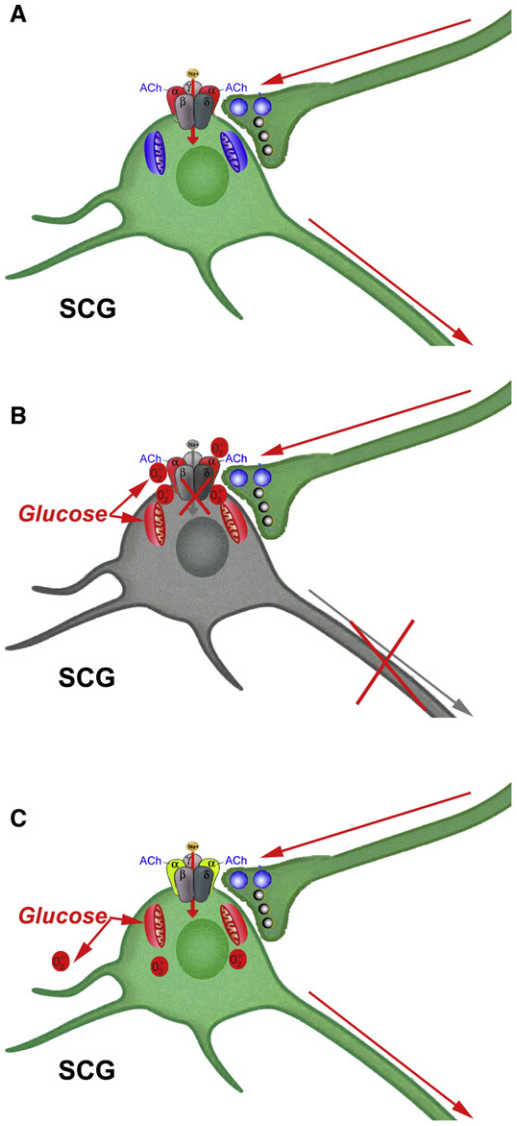

Figure 1. ROS Action on Sympathetic Neurons

(A) Preganglionic efferents of the sympathetic nervous system activate sympathetic neurons of the superior cervical ganglia (SCG) via activation of nictotinic acethylcoline receptors (nAchR). These postsynaptic receptors have various subunits: α, β, δ, γ. SCG efferents affect various peripheral tissue functions, including increasing heart rate, thermogenesis, and liver gluconeogenesis.

(B) During sustained hyperglycemia, glucose-generated free radicals (O_2^{o-}) oxidize cysteine residues in the α subunit (red) of the nAchR through which they diminish sympathetic synaptic transmission and trigger consequent alterations in peripheral tissue function. Glucose-generated ROS may arise from within SCG neurons via substrate oxidation or from extracellular sources.

(C) Mutating the a subunit of the nAchR (yellow) by eliminating cysteine residues prevents free-radical-induced impairment of nAchR signaling allowing continued synaptic transmission despite hyperglycemia-induced free-radical generation.

The paper by Campanucci et al. assigns a critical role for ROS in impairing sympathetic synaptic transmission. They found that treatment of neurons with antioxidants, α-lipoic acid and catalase, during hyperglycemia, prevented synaptic depression, thus indicating that ROS formation in hyperglycemic conditions is an important regulator of neuronal function. Whether the ROS affecting nAchRs are arising from within SCG neurons or extracellularly (Figure 1) is a critical question that needs to be resolved to better understand therapeutic potentials of the findings.

In recent years, it has become more evident that ROS are not merely harmful by-products of substrate oxidation, but also play vital roles in regulating neuronal responses and related behaviors as well as controlling peripheral tissue function (Andrews et al., 2008; Benani et al., 2007; Jaillard et al., 2009). It is tempting to draw some comparison between the effects of ROS on the final output component of the sympathetic nervous system (Campanucci et al., 2010) to ROS-regulated neurons in central regions of the brain presynaptic to the sympathetic nervous system (Elias et al., 1998). The mediobasal hypothalamus provides an important input for the autonomic nervous system (Elmquist et al., 1999). A subpopulation of neurons in the arcuate nucleus that produce pro-opiomelanocortin (POMC) are critical mediator's of leptin's effect on feeding behavior, energy expenditure, and the sympathetic nervous system (Gao and Horvath, 2007). These cells are connected with thoracic, preganglionic sympathetic neurons (Elias et al., 1998; Elmquist et al., 1999), and their activity is supported by

high ROS levels (Benani et al., 2007; Andrews et al., 2008; Horvath et al., 2009; Jaillard et al., 2009). Repeated activation of POMC neurons and related behaviors and autonomic adaptations occur daily. Short-term ROS peaks appear to be fundamental for evoking a proper behavioral, endocrine, and autonomic response to nutrient intake and are likely to be associated with short-term peaks of ROS generation in sympathetic neurons as well. On the other hand, prolonged exposure to hyperglycemia-triggered ROS clearly impairs sympathetic neuronal functions and outflow due to oxidation of the Cys residue of cholinergic receptors (Campanucci et al., 2010). Whether similar impairments of receptor activation (not necessarily cholinergic receptors) occur in POMC neurons in response to sustained ROS generation, when an animal is on high fat diet for example, is a highly relevant question to pursue.

In summary, the observations of Campanucci et al. (2010) shed new light on the etiology and offer potential new therapeutic approaches for diabetic neuropathies. Questions remain, however, regarding the source of ROS that impair nAchRs function: whether ROS are from intracellular or extracellular origin and whether ROS are the product of glucose oxidation or emerge from other metabolic processes. Further work is also needed to clarify whether effects of neuropathies promoted by diabetes influence hepatic glucose production and output and how restoration of sympathetic outflow would impact these critical processes.

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Alternative Splicing Disabled by Nova2

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Disabled-1 is a key signaling molecule in the Reelin pathway that plays a critical role in neuronal migration and positioning during brain development. In this issue of *Neuron*, Yano et al. demonstrate that the neuron-specific RNA binding protein Nova2 contributes to neuronal migration by regulating alternative splicing of disabled-1.

Neuronal migration and positioning in the developing brain is a complicated process that requires well-orchestrated interactions among migrating neurons, radial glial cells, and postmigration neurons. The Reelin pathway controls neuronal migration in the developing cortex, cerebellum, and hippocampus, and it has been implicated in human brain disorders such as lissencephaly, schizophrenia, bipolar disorder, autism, and temporal lobe epilepsy. Genetic and biochemical studies have helped elucidate the biochemical mechanisms responsible for Reelin signaling (for review, see Rice and Curran 2001; Ayala et al., 2007). Reelin is a protein ligand that binds two receptors: apoER2 and VLDLR. Binding of Reelin to these receptors triggers tyrosine phosphorylation of disabled-1 (Dab1) by Fyn and Src. Tyrosine phosphorylated Dab1 recruits downstream signaling molecules including the SH2/SH3 domain-containing adaptor proteins, Crk and CrkL, as well as other signaling components, before it is degraded by the ubiquitin-proteasome pathway. These adaptor proteins induce cytoskeletal changes and other cellular responses necessary for appropriate migration and positioning of neurons. Therefore, Dab1 plays a central role in Reelin signaling, and failure of either expression or phosphorylation of Dab1 leads to severe defects in neuronal migration, similar to those observed in the absence of Reelin or Reelin receptors. In this issue of *Neuron*, Yano et al. present a novel mechanism of Dab1 regulation involving alternative splicing by Nova2.

Nova2 is an RNA-binding protein specifically expressed in neurons. It was identified as an autoantigen in paraneoplastic opsoclonus myoclonus ataxia (POMA) (Yang et al., 1998), a neurologic